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Spin-labelled diketopiperazines and peptide–peptoid chimera by Ugi-multi-component-reactions†

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For the first time, spin-labelled coumpounds have been obtained by isonitrile-based multi component reactions (IMCRs). The typical IMCR Ugi-protocols offer a simple experimental setup allowing structural variety by which labelled diketopiperazines (DKPs) and peptide–peptoid chimera have been synthesized. The reaction keeps the paramagnetic spin label intact and offers a simple and versatile route to a large variety of new and chemically diverse spin labels.

Electron paramagnetic resonance (EPR) spectroscopy is a well-established method which has become a strong tool for determination of structural features in particular in biomolecules as well as resolving interactions of membrane–peptide interactions.[1–5] In EPR studies in the field of protein structural biology, the spin label bearing moiety is generally incorporated by attachment to amino acid side chains. The suitability of two of the most popular spin probes **1** (methane thiosulfonate spin label, MTSSL) and **2** (4-amino TEMPO) has been demonstrated in numerous studies, in which they are attached to *e.g.* the cysteine thiol group and are often proven to have minimum impact on the secondary and tertiary structure of proteins (see Fig. 1 for a selection of spin labels).[6,7]

Less frequently, modified (unnatural) amino acids are used, which can be introduced during peptide synthesis or recombinant protein production. These are mostly the prochiral TOAC **3** and chiral TOPP **4**.[8,9] Similarly, spin-labelling of small-molecule ligands that bind to proteins is achieved most often by attaching TEMPO-derivatives where possible. However, all these protein and small-molecule modifications provide single case solutions only, necessitating an inevitable amount of experiments to access a variety of products.[10] Here, we show,

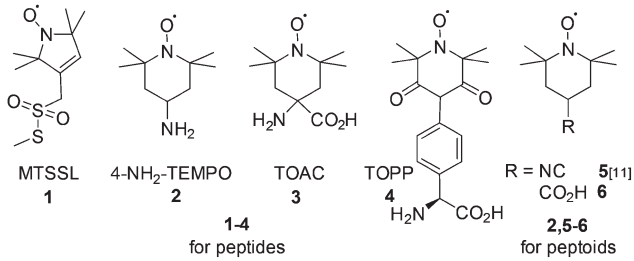

Fig. 1 Spin labels suitable for peptides and peptoids.

to the best of our knowledge for the first time, that multi-component reactions such as isonitrile-mediated Ugi-reactions (see Scheme 1) can serve as a synthetic tool to obtain spin-labelled products starting from TEMPO-derivatives **2**, **5–6** (*cf.* Fig. S1 and S2† for EPR spectra) offering a broad portfolio of variations with reduced synthetic endeavor when compared to consecutive chemical synthesis.[11] We also demonstrate that the precondition for this versatile tool is that the spin label is quite stable under conditions of the Ugi-reaction and peptide coupling protocols.

The Ugi-reaction may seem intimidating in the context of peptide-modifying reactions as one inherently has to choose which of the four Ugi-reaction components the spin label may be bound to. Modifications can be done on the carboxylic acid, the carbonyl, the isonitrile, or the amine component. The examples presented here have the spin label on the amine, the carboxylate and the isonitrile building block, the resulting Ugi-products will be transferred to diketopiperazines (DKP) and peptide–peptoid chimera to demonstrate the utility of this approach.[12,13]

Along with each synthesis step, CW EPR spectra of chosen samples are discussed and analyzed in terms of their hyperfine coupling, solvent effect and spectral line shape. All the reported values are obtained *via* rigorous spectral simulation using the MATLAB-based Easyspin software package.[14]

For the synthesis, no variations of classical protocols were necessary to obtain spin-labelled Ugi-products **8a–d** and **9a–h**

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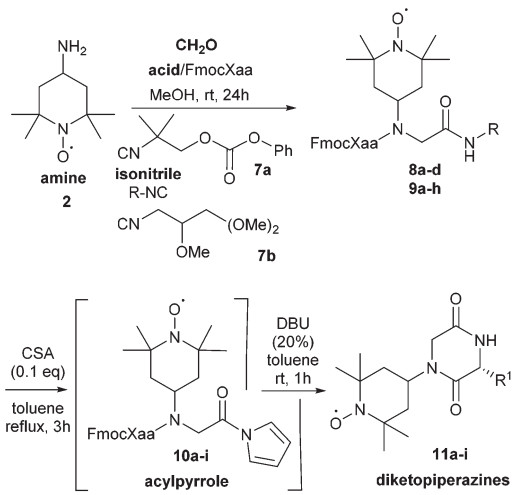
(Scheme 1, Fig. 2, for **9a–h** see ESI†). The Ugi-reactions were carried out with convertible isonitriles, either **7a**, Fukuyama's isonitrile[15] or IPB **7b**,[16] which allow further functionalization of the terminally formed amide bond. In initial experiments we worked with isonitrile **7a**, because the secondary amide in Ugi-product **8a** offers the possibility for formation of *N*-acyloxazolidinones, upon which the amide bond is highly susceptible to nucleophilic attack. Simultaneously, the nucleophile is formed intramolecularly upon basic cleavage of the Fmoc-protecting group leading to spontaneous formation of diketopiperazines **11**. Unfortunately, we could not establish conditions to achieve this goal. Therefore, we turned to IPB **7b** as the convertible isonitrile. For the synthesis of diketopiperazines **11** we intended to use a sequence which was developed by us earlier.[17,18] In a consecutive Ugi4CR/deprotection + activation/cyclization (UDAC) -strategy, this short sequence can accomplish the appropriate products **11a–i** (Scheme 1, Fig. 2). In practice, after formation of the Ugi-products, the corresponding intermediary acylpyrroles **10** are formed in the presence of 0.1 eq. camphor sulfoinic aicd (CSA) under reflux. A short workup to neutralize the reaction mixture is followed by a DBU-mediated cleavage of the Fmoc-group. Spontaneously, cyclization to diketopiperazines **11a–i** can be observed in the range of 50–70% yield overall. Peptoids **8c**, **d** have not been used for further derivatizations yet, their usage in the synthesis of chimera will be reported in due course.



Scheme 1 Synthesis of diketopiperazines **11** using a Ugi4CR/deprotection + activation/cyclization (UDAC) -strategy.

For the synthesis of **11i**, isobutyric aldehyde was used instead of formaldehyde, the yields were comparable, however, the reaction only afforded a diastereomeric mixture, as expected.

Gratifyingly, no disproportionation/decay of the radicals could be observed under the reaction condition. Even reflux conditions during formation of the acylpyrroles **10** did neither lead to any significant changes in shape nor in intensity of the typical three lines pattern of nitroxides. Also, storage of all spin-labelled compounds is possible for several weeks at 6 °C.

It is interesting to EPR-spectroscopically compare the Ugi intermediate **8b** and its corresponding DKP **11a**. Experimental spectra together with simulations are given in Fig. 3a and b. Both spectra show the characteristic three line pattern of nitroxides. The chemical stability of the six-membered nitroxide radicals during synthesis and after treatment in acidic or basic condition has been tested by a quantitative assessment of the spin label concentration (not shown). The corresponding simulated spectra are given in Fig. 3a and b. The Ugi intermediate **8b** has an isotropic hyperfine splitting, A(^{14}N), of

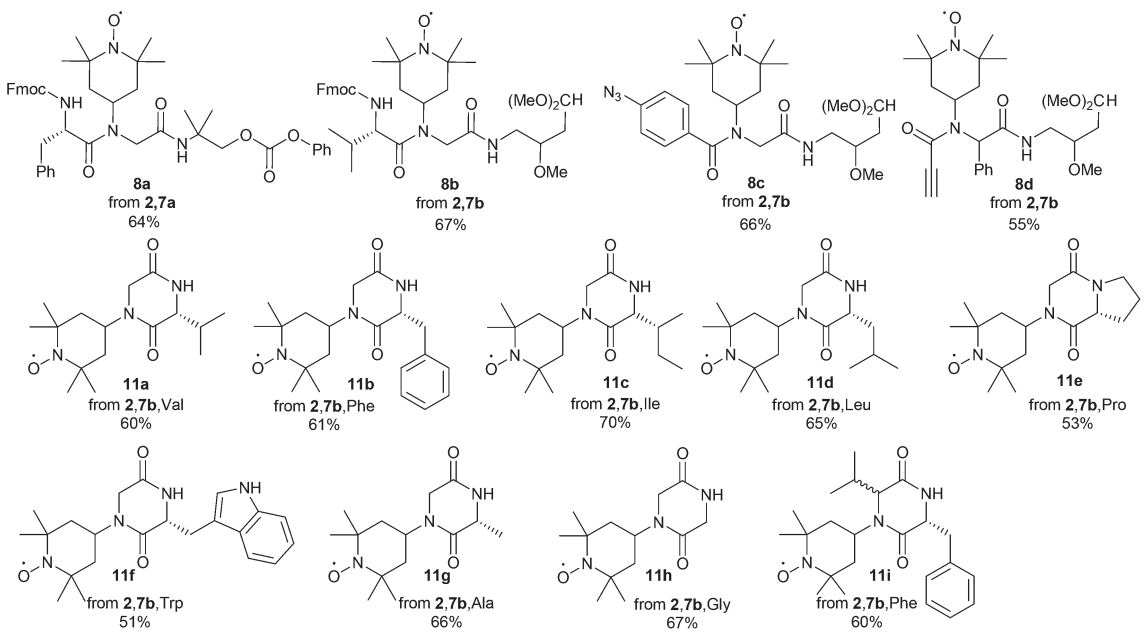


Fig. 2 Ugi-products **8**, diketopiperazines **11** with spin-labels attached.

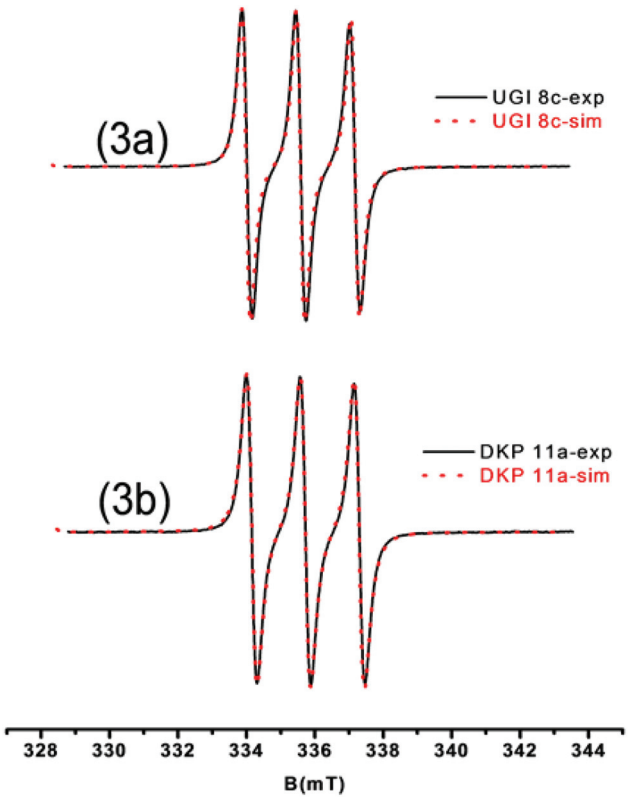


Fig. 3 Room temperature CW EPR spectra (black solid line) and their spectral simulations (red dotted line) of Ugi intermediate **8b** and its corresponding DKP **11a**.

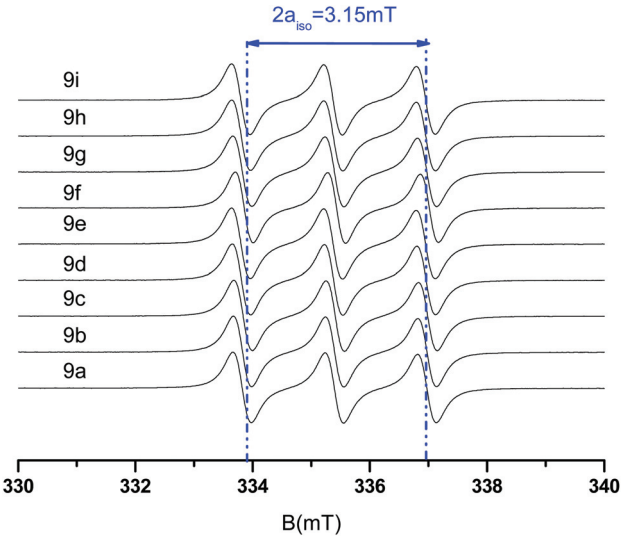


Fig. 4 Room temperature CW EPR spectra of final DKPs **11a–i** (CH_3CN).

44.7 MHz and for the final DKP **11a**, we found a similar value of 44.0 MHz. The rotational correlation time (τ_c) of the Ugi intermediate obtained as 0.03 ns which is in the range of fast rotational motion that is expected for a spin label attached to a medium-sized molecular backbone. As expected for the DKP product, the obtained τ_c value was smaller than for the corresponding Ugi compound, which is due to the cleavage of protecting bulky FMOC group, rendering rotational motions for the spin-labelled DKP ten times faster. For both cases we find an isotropic *g*-value of 2.005 which is in the typical range of isotropic *g*-value for nitroxides.[19] The complete set of recorded spectra for all final DKP compounds, dissolved in acetonitrile, is presented in Fig. 4. Details on spectral simulation are given in Table S1.†

Using EPR spectroscopy we can observe even slight structural differences between DKPs. The two DKPs **11a** (from valine) and **11b** (from phenyl alanine) have a very similar chemical structure. The different substitution patterns do not afford a difference in the hyperfine coupling values of the nitroxide (A(^{14}N)) but rather makes **11a** rotate faster by a factor of 1.6, according to the simulations. Therefore, one can utilize EPR spectroscopy also as a screening method to detect slight structural differences, which can then be further analysed in detail (CW EPR spectra of DKP, **11b** are given in Fig. S3†).

To test for the effect of different solvents on the DKP products, and as potential use of spin-labelled DKPs for biophysical or bioanalytical applications mandates water as a solvent, the DKP **11a** was also studied in aqueous solution (Fig. S4†). As can be seen, the A(^{14}N) deviates and is about 3.8 MHz higher in water as compared to acetonitrile. It is well established that the hyperfine splitting is an excellent probe of the environmental polarity, *e.g.* of the solvent or of nanophase-separation in polymeric substances.[20–24] Spectral line shapes are also affected by the solvent of choice. We observed a dominant contribution from Lorentzian line shape rather than Gaussian in case of using acetonitrile while we had a higher Gaussian contribution in water. Overall, rotational dynamics of the molecules are slowed down in water. This effect can be attributed to the formation of hydrogen bonds between solvent molecules and the nitroxide moiety or other H-bonding groups (amines, carbonyls) of the probes.

To expand the functional scope of spin-labelled substances, peptide–peptoid chimera have been synthesized.[25] The examples presented in Fig. 5 have been obtained using three different Ugi-amenable TEMPO derivatives (**2**, **5**, **6** in Fig. 1) to incorporate the spin label *via* the carboxylic acid, the amine and the isonitrile moiety, respectively.

To achieve the synthesis of the chimera **12–14**, amino acids have been chosen as the corresponding amine and carboxylic acid counterparts, the other functionalities have been protected by classical means (Cbz, Boc). In all cases, formaldehyde has been used as the carbonyl component to avoid formation of stereoisomers (Fig. 5). These examples leading to C- and N-terminal as well as side chain modified products clearly indicate the very flexible utilization of nitroxide-based spin labels within the Ugi-reaction. It leads to a potentially very large library of desired products in a single step only. The incorporation of two spin-labelled moieties can be achieved *via* a one-pot setup, in which two subsequent Ugi-reactions can lead to double-labelled **16**. The reaction partner for the

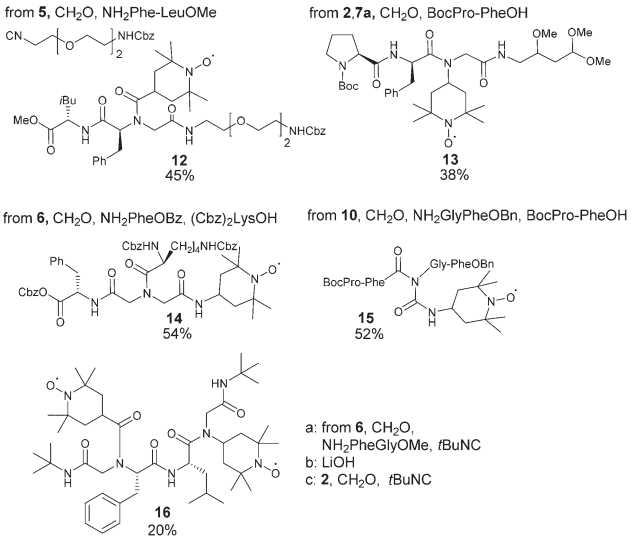


Fig. 5 Spin-labelled peptide–peptoid chimera **12–16**.

second Ugi-reaction, however, needs to be prepared intermediary by a saponification to provide a carboxylic entity.

The corresponding EPR spectra of peptide–peptoid chimera, are given in Fig. 6. Spectral properties obtained from simulations are summarized in Table 1.

Peptides **14** and **15**, have spin labels incorporated at the end of chain, so the spin labels experience a higher degree of rotational freedom than in peptides **12** and **13** that are spin-labelled in the middle of the chain.[26] As a result, peptides **14** and **15** rotate faster and have shorter correlation times compared to two other peptides. Peptide **15** has the fastest rotational motion owing to its smaller size compared to the rest of the studied molecules.

Simulated hyperfine values are very similar to those of the Tempo derivatives from which the peptides were derived. The A(^{14}N) and isotropic *g*-values indicate the possible presence of one or two hydrogen bonds attached to nitroxyl moiety of the spin label.[20–24] Peptide **14** was not well soluble in water, so it was dissolved in methanol and then measured. As a result it displays smaller A(^{14}N) values and broader lines (due to larger amounts of dissolved molecular oxygen in methanol) as compared to the rest of peptides which were measured in water. The isotropic *g*-value remains typical for nitroxides.

Since highly resolved spectra with detailed information can be obtained at higher frequencies/magnetic fields, EPR measurements of a synthesized doubly spin-labelled peptide, biradical **16**, were performed at higher frequency of 34 GHz (Q-band, magnetic field B ~1.1 T), in addition to the measurements at X-band (9.4 GHz, B ~0.35 T).

Comparing X- and Q-band spectra reveals a completely different (apparent) rotational dynamic appearance for this peptide at Q-band frequency (*cf.* Fig. 7). Simulations show that the spectrum contains two components; a three line spectrum of the two individual radicals not interacting with each other and a five line biradical component which stems from the same molecules but reflects the fraction of the ensemble in which the two radical centers show Heisenberg spin-exchange interaction. This means that the radicals collide with an exchange frequency, probably due to the conformation of the

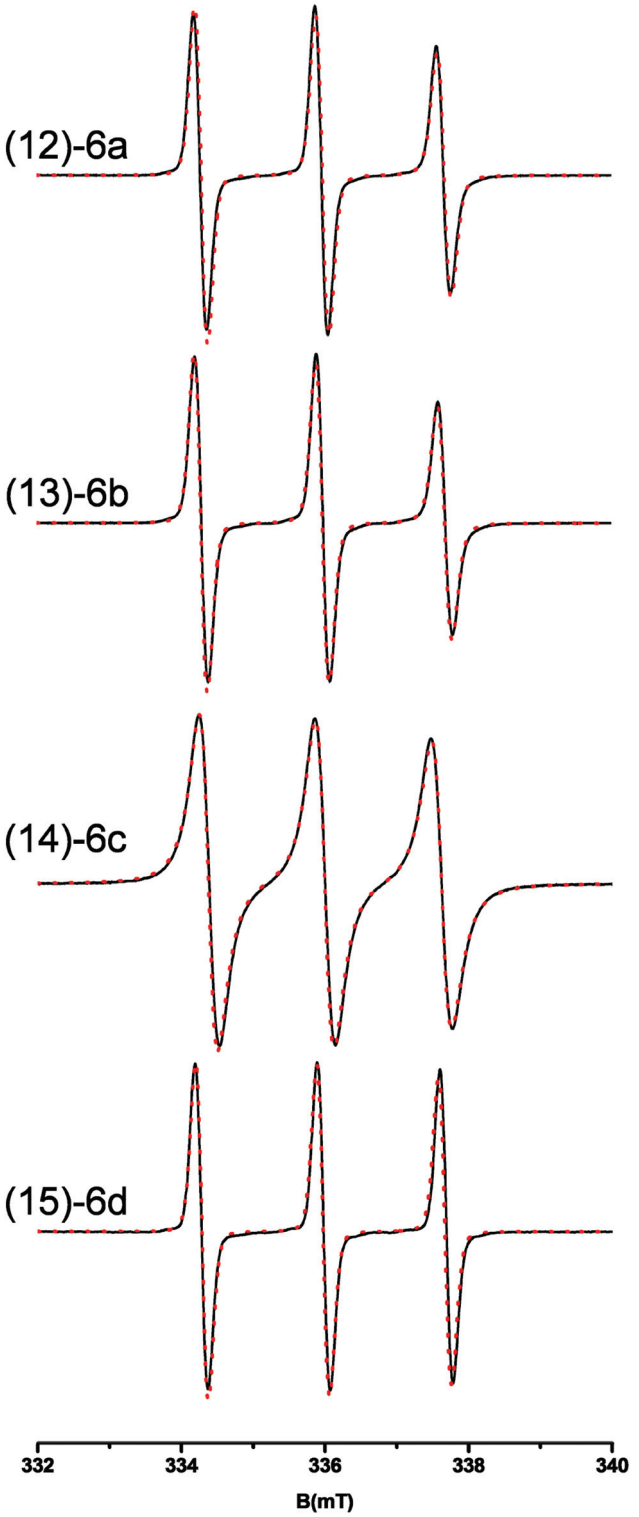


Fig. 6 Experimental (solid black line) and simulated (red dotted line) spectra of spin-labelled peptides **12–15**.

Table 1 Spectral properties of synthesized spin-labelled peptides. Rotational correlation times are in nanoseconds-ns- and hyperfine splittings in MHz[a]

#Peptide	τ_c	A(^{14}N)	g_{iso}
12	0.18	47.50	2.005
13	0.26	47.60	2.005
14	0.14	45.60	2.005
15	0.046	47.50	2.005

[a] All peptides were measured in water, except #**14**, which was measured in methanol.

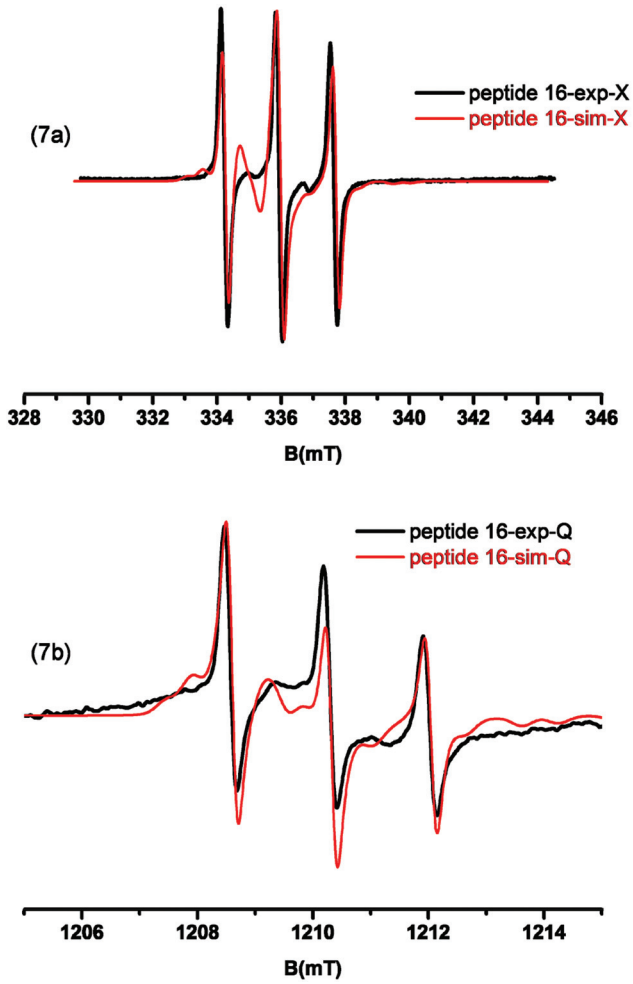


Fig. 7 EPR measurements of doubly spin-labelled peptide **16** in water. In a global simulation approach, the simulation parameters of the better resolved Q-band spectrum (**7b**), was used for simulating the X-band spectrum (**7a**).

peptide in solution. As can be seen from Fig. 7, the biradical presence is more pronouncedly observable at Q-band. The biradical spectral contribution was found to amount to as much as 35% of the overall spectrum. The isotropic part of the electron-electron spin–spin interaction can be quantified by the exchange interaction frequency (Heisenberg spin exchange coupling constant J_{iso}), which was found from the simulations to be ~13 MHz.

Conclusions

To summarize, we for the first time presented a synthetic route to spin-labelled compounds *via* isonitrile-mediated multicomponent reactions. To reveal the flexibility of this approach various peptides, peptide–peptoid chimera and diketopiperazines varying the position of the spin-label have been synthesized. The reactions conditions of the Ugi-reaction were found to comply with the radical nature of the label, in all cases quantitative EPR-analysis was possible and revealed even subtleties of the different molecular structures. We envision that the Ugi-reaction will become a valuable and simple synthetic tool to provide compounds of some complexity for EPR analysis, *e.g.* for screening approaches in biomedically relevant ligand binding studies.

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